MECHANISMS OF REMOVAL OF INJECTED MICROORGANISMS FROM THE AMERICAN OYSTER, CRASSOSTREA VIRGINICA (GMELIN)[1, 2, 3]

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Extensive studies on the amoebocytes[4] of oysters have elucidated their importance in digestion, but these cells have not been extensively studied as regards their role in defense. It is clear that in this filter-feeding mollusc, amoebocytes move freely through the gut epithelium and engulf partially digested particulate food (Yonge, 1926; Takatsuki, 1934). Intracellular digestion follows and the products of digestion are presumably made available to all the cells of the body. In other Mollusca, complex digestive organs are present and intracellular digestion plays a minor role (Yonge, 1937). In such animals amoeboid cells associated with certain tissues and organs act as specialized defensive agents (Metchnikoff, 1901; Cuénot, 1914).

Stauber (1950) demonstrated that carbon particles (India ink) injected intracardially in the American oyster, *Crassostrea virginica,* are taken up by phagocytes and removed from the tissues as these cells migrate through epithelia to the exterior. However, intracardially injected red blood cells are eliminated both by migration of phagocytes and by intracellular digestion (Tripp, 1958). These studies suggest that two separate defense mechanisms are involved in the oyster, and a study of the relative importance of these processes in relation to injected microorganisms serves as the basis for this report.

MATERIALS AND METHODS

1. *Maintenance of oysters (C. virginica)*

The oysters used in these experiments were obtained from either Delaware Bay or the Navesink River at Red Bank, New Jersey. They were thoroughly scrubbed with a wire brush to remove encrusting organisms and mud, and then were maintained in groups of eight or fewer in three-gallon Pyrex battery jars containing one gallon of sea water. This water was of the same salinity as that from which the oysters were originally obtained and was changed twice daily. All aquaria were constantly aerated and were maintained at 17 ± 1° C.

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[4] The terms amoebocyte, phagocyte and leukocyte are used interchangeably. The cells referred to are found free in the blood and are both amoeboid and phagocytic.

In preparation for intracardial injection a small hole was made (using a coarse wood rasp or a ¼" electric drill) in the left valve of the shell, directly over the heart. Once the nacreous layer had been penetrated, the opening was carefully enlarged with forceps until the entire heart was visible. A scalpel incision through the mantle and pericardial membrane then exposed the heart. These wounds began to heal within a few days and many oysters formed new shell before they were sacrificed.

Preliminary experiments demonstrated that the adductor muscle was also suitable for injection. The muscle bundles are bathed by extensive blood channels which drain directly into systemic vessels so that material injected into the muscle very quickly reaches the systemic circulation. In preparation for intramuscular injections both valves of the shell dorsal to the adductor muscle were filed so as to expose the muscle, caution being taken to avoid injury to the subjacent tissue.

Prior to injection each oyster was appraised as to its general physiologic condition in terms of strength and regularity of heart beat, frequency of shell closure, and ejection of fecal strings. Animals which did not appear normal were discarded.

2. *Maintenance of microorganisms*

All microorganisms selected for injection could be recognized by colonial characteristics in agar pour plates and, thus, could be distinguished from the endogenous bacterial flora of oysters.

A strain of *Bacillus cereus* var. *mycoides* (*B. mycoides*) was obtained from the Institute of Microbiology, Rutgers, the State University. Cultures of *Staphylococcus aureus, Escherichia coli, Pseudomonas fluorescens* and *Saccharomyces cerevisiae* were obtained from the Department of Bacteriology, Rutgers, the State University. *Flavobacterium invisibile* was isolated from oyster heart blood. Microorganisms were subcultured routinely on nutrient agar slants, but for purposes of preparing inocula, organisms were grown as outlined in Table I.

All cultures in liquid media were harvested by centrifugation and washed three times with 15 ml. of sterile 0.85% saline. Spores were harvested from agar plates

TABLE I

Media, incubation times and temperatures for various microorganisms used in injecting oysters

Organism	Medium	Incubation	
		Temp. (°C.)	Time (Days)
Bacillus cereus var. *mycoides*		25	5
spores	Nutrient agar (dist.)*	then 5	7
vegetative cells	Nutrient broth (dist.)	25	3
Staphylococcus aureus	Nutrient broth (dist.)	37	1
Escherichia coli	Nutrient broth (dist.)	25	1
Pseudomonas fluorescens	Nutrient broth (dist.)	25	1
Flavobacterium invisibile	Nutrient broth (sea)	25	1
Saccharomyces cerevisiae	Sabouraud broth (dist.)	25	2

* Difco Bacto-nutrient agar, broth or Sabouraud broth rehydrated with distilled or sea water.

and were similarly washed. Final suspensions of organisms were prepared in autoclaved sea water of the same salinity as that in which the oysters were maintained. Prior to injection, the concentration of microorganisms in each inoculum was estimated by dilution plate counts.

3. Injection procedures

Spores and vegetative cells of *B. mycoides,* and vegetative cells of *Staph. aureus, F. invisibile* and *S. cerevisiae* were injected intracardially. Vegetative cells of *E. coli* and *P. fluorescens* were injected into the adductor muscle.

All injections were done with a 1.0-ml. tuberculin syringe fitted with a 27-gauge (¾") or a 30-gauge (1½") needle. Each oyster was placed in sea water and observed with a binocular dissecting microscope while the needle was inserted directly into the ventricle or adductor muscle; 0.2 ml. of inoculum was injected over a period of several seconds. Oysters received 10^5–10^9 microorganisms, depending on the species of organism and the nature of the experiment. Withdrawal of the needle allowed the wound to close and little of the inoculum was lost. Animals from which excessive leakage occurred were discarded. Each inoculated oyster was passed through six changes of fresh sea water, during which the area over the injection site was flushed gently with a pipette to remove extraneous microorganisms. The oysters were then returned to aquaria and maintained as described above.

4. Sampling procedures

Two types of experiments were performed in order to determine the fate of injected microorganisms. In one group of experiments oysters which had received intracardial injections of *B. mycoides* or *S. cerevisiae* were relaxed, fixed, and prepared sections were examined microscopically. In the second group of experiments tissues from experimental oysters were cultured (at intervals) to recover microorganisms.

a. Sections of inoculated oysters

Preliminary experiments showed that tissue shrinkage due to fixation and dehydration could be minimized by relaxing intact oysters in 0.4 *M* magnesium sulfate at 4° C. for several hours prior to fixation of the whole oyster in Bouin's solution overnight. Ten oysters were injected intracardially with a suspension of *B. mycoides* and one animal was sacrified at each of the following intervals after injection: 1 hour, and 1, 2, 4, 5, 8, 9, 12, 16 and 20 days. Similarly six oysters were injected intracardially with a suspension of *S. cerevisiae* and one animal was sacrificed at each of the following intervals thereafter: 1 hour, and 1, 2, 3, 4 and 12 days. Two or three control oysters were injected with equal volumes of sterile sea water and sacrificed at suitable intervals in each experiment. Examination of control animals disclosed no histological abnormalities or microorganisms.

Following initial fixation the soft parts were removed from the shell, divided transversely into six approximately equal parts and placed in fresh Bouin's solution for 1–4 hours. These blocks of tissue were then washed, dehydrated, cleared, imbedded in paraffin, sectioned at 10 μ and stained with Gram's tissue stain. Two sections were taken from each block, one representing the anterior edge of the tissue and one from the center of the tissue. Thus, for each animal 12 transverse sections were obtained at regular intervals along the anteroposterior axis.

Certain vessels, sinuses and tissues were regularly examined for microorganisms under high dry and oil immersion objectives. These included: heart, anterior and posterior aortae, large arteries of the visceral mass, subepithelial blood sinuses of the gut, sinuses between digestive diverticula, dorsal and ventral circumpallial arteries, proximal mantle vessels, medial and lateral gill axis sinuses, vertical gill vessels, palp arteries and the blood spaces of the adductor muscle and kidney. In addition, the following epithelial areas were examined for microorganisms: walls of the alimentary canal at all levels, walls of twenty of the digestive diverticula, inner and outer aspects of the palps, outer aspect of the mantle, nephridial tubules, gonaducts, epicardium and pericardium.

b. Cultivation of microorganisms from tissues

Groups of 20–25 oysters received injections of microorganisms suspended in sterile sea water, while 6–8 controls were injected with sterile sea water only. Estimates of the numbers of microorganisms in heart blood, mantle, visceral mass and in aliquots of homogenized whole oysters were made at intervals of one hour, one day, two days, and irregularly thereafter for 6–50 days.

At each interval three experimental and one control oysters were tested for microorganisms according to the following scheme. Each heart yielded 0.1–0.5 ml. of blood which was added to 4.5 ml. of sterile distilled water. Portions of tissue, approximately 0.1 ml. in volume, were removed from the mantle and visceral mass and ground separately in Ten Broeck tissue grinders containing 5.0 ml. of sterile distilled water. The remaining soft parts were homogenized in a Waring Blendor containing 20 ml. of sterile distilled water, and the actual volume of the soft parts was determined by displacement. One-tenth ml. of the material from each tissue or homogenate sampled was placed in each of three Petri dishes, melted agar added and the plates incubated at the optimum temperature for the species

Table II

Media, incubation times and temperatures for bacteriologic tests of oyster tissues

Organism	Medium	Incubation	
		Temp. (°C.)	Time (Hours)
Bacillus cereus var. *mycoides*			
spores	Nutrient agar (dist.)*	30	24
vegetative cells	Nutrient agar (dist.)	30	24
Staphylococcus aureus	Nutrient agar (dist.)	37	24
Escherichia coli	Nutrient agar (dist.)	37	24
Pseudomonas fluorescens	Nutrient agar (sea)	25	48–72
Flavobacterium invisibile	Nutrient agar (sea)	30	18
Saccharomyces cerevisiae	Sabouraud agar (dist.)	30	18–24

* Difco Bacto-nutrient agar or Sabouraud agar rehydrated with distilled or sea water.

of microorganism being studied. Individual colonies were counted, averaged and the number of viable microorganisms per ml. of sample was calculated. Using these data, and estimates of the volumes of mantle and visceral mass (respectively 30% and 40% of the soft parts) derived from previous experiments, it was possible to estimate the total number of viable microorganisms in these organs. Results are expressed as the mean values from three oysters. In general, values obtained were in close agreement and only rarely were extreme variations noted. Since there was no suitable method of estimating the total volume of oyster blood, the number of microorganisms recovered from blood samples is expressed per ml. of heart blood sampled.

RESULTS

1. Observations based on tissue sections following injection of yeast cells

Masses of yeast cells seemed to occlude main arteries immediately after intracardial injection. Phagocytosis began immediately and by 24 hours phagocytes contained approximately 95% of yeast cells visible in sampled areas of tissue sections. Tissue sections from the oyster fixed at 24 hours showed that most leuko-

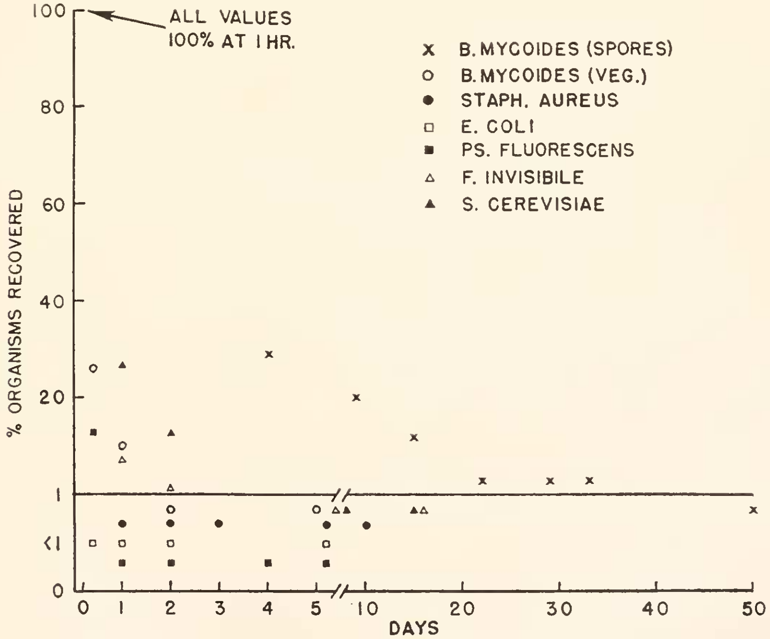

FIGURE 1. Survival of injected microorganisms per ml. sample of oyster heart blood taken at intervals up to 50 days. Base line samples at one hour represent 100% recovery, and subsequent results are expressed proportionately. Each point represents the mean number of microorganisms from three oysters sampled. Points in the area labeled "< 1" are not quantitative measurements, but indicate that small numbers of bacteria were detected for several days following injection. The last point shown for any one microbial species indicates the termination of a sampling series.

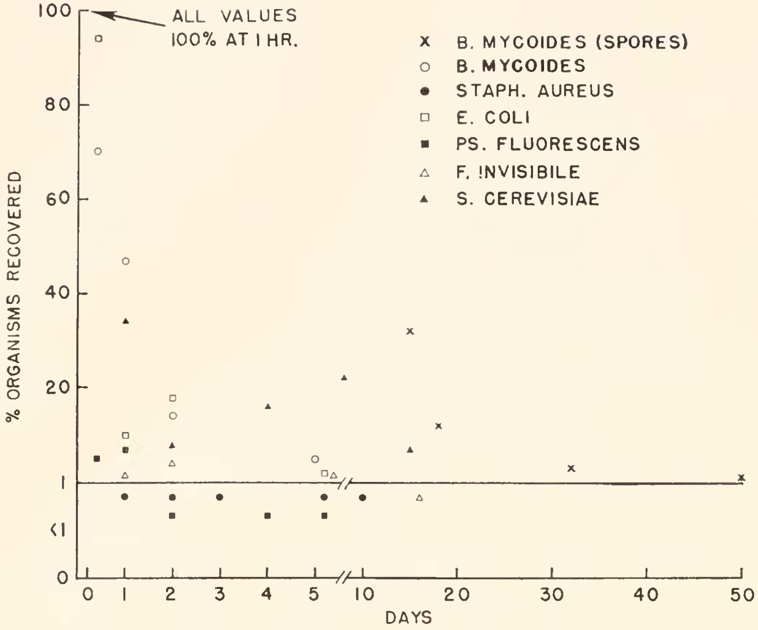

FIGURE 2. Survival of injected microorganisms in samples of oyster mantle tissue at intervals up to 50 days. Base line samples at one hour represent 100% recovery, and subsequent results are expressed proportionately. Each point represents the mean number of microorganisms from three oysters sampled. Points in the area labeled "< 1" are not quantitative measurements, but indicate that small numbers of bacteria were detected for several days following injection. The last point shown for any one microbial species indicates the termination of a sampling series.

cytes contained 2–4 yeast cells, some 8–10 yeast cells, and a few as many as 15–20 yeast cells. It was also evident that appreciable numbers of yeast-laden leukocytes had begun to migrate from blood vessels into adjacent tissues.

During the second day phagocytosis and migration progressed with the result that although individual yeast cells were still present in smaller vessels, large masses of yeast cells were no longer present in the main vessels. Phagocytes containing yeast cells were first seen in the epithelium lining the gut and the digestive diverticula at 48 hours. By the third and fourth day a relatively massive investiture of yeast-bearing phagocytes was evident in the epithelium of the gut, digestive diverticula, palp and mantle, but rarely were such leukocytes found in the epithelium of the gills, kidney, pericardium or gonaducts. Few yeast cells remained in any of these tissues at 12 days post-injection and it appeared that the majority of yeast cells had been eliminated by the migration of yeast-bearing leukocytes to the exterior.

Substantial variations observed in the morphological and tinctorial properties of a small proportion of yeast cells were thought to represent the effects of intracellular digestion. The normally oval or circular outline of some yeast cells became irregular as early as the fourth day post-injection, and instead of appearing a uniformly deep blue these yeast cells were mottled or very pale blue. Ultimately, only faintly staining globules, thought to represent the remains of yeast cells, were seen intracellularly. Morphologically-altered yeast cells were rarely seen outside of phagocytes.

2. *Observations based on tissue sections following injection of B. mycoides*

Suspensions of *B. mycoides* injected intracardially were composed mainly of short chains of vegetative cells which could be detected in Gram-stained tissue sections up to the ninth day. Examination of heart blood smears and tissue sections taken one hour post-injection clearly showed that large numbers of bacteria had already been phagocytized. Tissue sections from the oysters fixed at one hour and later disclosed that phagocytized bacteria were transported within a few hours from major blood vessels into adjacent tissues, while non-phagocytized bacteria remained in the blood vessels. Within 2–5 days post-injection most of the phagocytized bacteria were removed from the oyster by phagocytes migrating through the gut epithelium, or through the inner or outer surfaces of the mantle.

Phagocytized microorganisms which failed to be eliminated via an epithelial

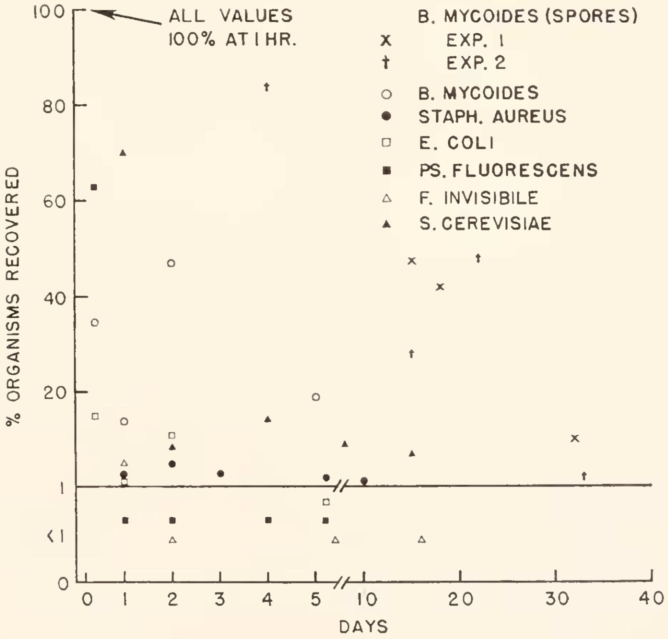

FIGURE 3. Survival of injected microorganisms in oyster visceral mass tissue at intervals up to 33 days. Base line samples at one hour represent 100% recovery, and subsequent results are expressed proportionately. Each point represents the mean number of microorganisms from three oysters sampled. Points in the area labeled "< 1" are not quantitative measurements, but indicate that small numbers of certain bacteria were detected for several days following injection. The last point shown for any one microbial species indicates the termination of a sampling series.

surface underwent vacuolization and loss of Gram-positive character, suggesting that they were dead or dying. Similar changes occurred among extracellular organisms during the first 48 hours after injection. Such degenerative changes were observed only very rarely in control (sea water) suspensions of bacteria maintained at room temperature for similar periods of time.

Except for local accumulations of leukocytes, the significance of which was difficult to evaluate, no distinct pathology was observed in sections of injected oysters.

3. Results of culturing living microorganisms from tissues

Recovery of viable microorganisms from samples of blood, organs and homogenized whole oysters resulted in two distinct groupings of data, one for bacterial spores and the other for vegetative bacteria or yeast (Figs. 1–4). While only a relatively small proportion of injected vegetative cells could be recovered and only within 24–48 hours after injection, bacteria injected as spores were recovered in relatively larger proportions over a period of several weeks. Although the inocu-

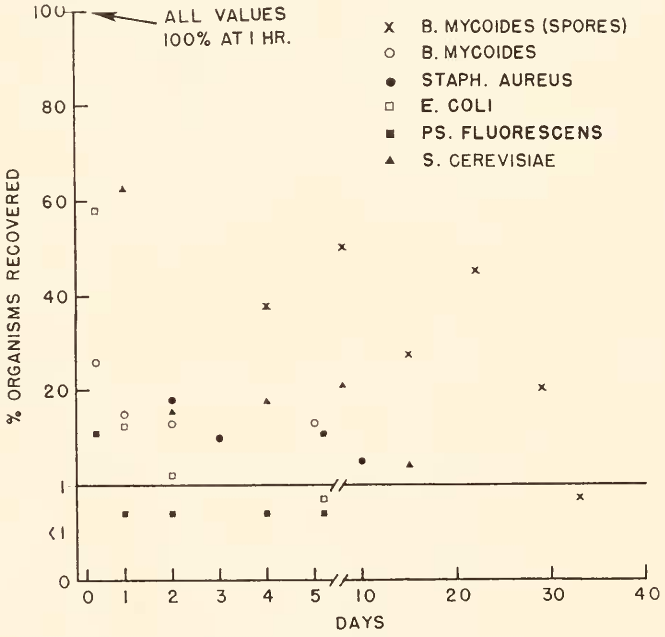

FIGURE 4. Survival of injected microorganisms in whole oysters homogenized at intervals up to 33 days. Base line samples at one hour are assumed to represent 100% recovery, and subsequent results are expressed proportionately. Each point represents the mean number of microorganisms of three oysters sampled. Points in the area labeled "< 1" are not quantitative measurements, but indicate that small numbers of certain bacteria were detected for several days following injection. The last point shown for any one microbial species indicates the termination of a sampling series. (Data for *F. invisibile* are omitted because of contamination of samples.)

lated microorganisms were not completely removed, there was no evidence of bacterial multiplication.

COMMENT

It is evident that injection of any of several foreign particles into the oyster elicits a basic cellular response. Studies reported here indicate that injected yeast cells or bacterial spores were disposed of by the oyster in the same manner as carbon particles (Stauber, 1950). In all of these cases particles became clumped in efferent vessels and large numbers of leukocytes accumulated around them; most particles were engulfed by leukocytes, removed from the circulation and subsequently transported through epithelial layers to the exterior. These processes are initiated immediately after injection, continue until most foreign particles have been removed and may be supplemented by intracellular and/or extracellular destruction. On the other hand, the oyster's defense against injected vegetative bacteria seems largely to be dependent upon intraphagocytic digestion. It is possible that oysters maintained under more favorable conditions (*i.e.,* in larger volumes of sea water) would eliminate injected microorganisms even more rapidly. It would be of interest to determine the fate of an oyster pathogen under such circumstances.

TABLE III

Method of removal	Present study			Tripp '58	Stauber '50
	Bact. spores	Yeast	Veg. bact.	R.B.C.	Carbon
Phagocytosis and migration	++++	++++	+	++	++++
Phagocytosis and digestion	N.O.	+	++++	++++	N.O.
Extracellular destruction	N.O.	N.O.	++	N.O.	N.O.

N.O.—not observed.

Table III summarizes qualitatively the relative importance of various processes in the disposal of injected particles.

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SUMMARY

1. The fate of injected bacterial spores, vegetative bacteria and yeast cells injected into the oyster, *Crassostrea virginica,* was followed histologically and by viability studies.

2. Tissue sections showed that yeast cells were phagocytized in blood vessels by leukocytes which migrate into surrounding tissues and later through epithelial layers to the exterior. A small proportion of yeast cells remaining in the tissues are digested intracellularly.

3. Histologic examination of oysters injected with living bacteria revealed that the microorganisms were rapidly destroyed both intracellularly and extracellularly before significant numbers of host phagocytes could migrate to the exterior.

4. Viability experiments demonstrated that when several species of non-spore-forming bacteria were injected, very few of these organisms could be recovered 48 hours later. However, a small proportion of these bacteria persisted in oyster tissues for several days.

5. Bacterial spores were removed from oyster tissues at a much slower rate than vegetative bacteria.

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